MITOCHONDRIAL DNA SEQUENCE VARIATION IN POPULATIONS OF THE NINE-BANDED ARMADILLO (*DASYPUS NOVEMCINCTUS)*

Diana Adams Elrod, B.S., M.S.

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APPROVED:

John V. Planz, Major Professor
Robert Benjamin, Committee Member
Gary Heidt, Committee Member
Gerard O'Donovan, Committee Member
Mark Shanley, Committee Member
Earl Zimmerman, Chair of the Department of Biological Sciences
C. Neal Tate, Dean of the Robert B. Toulouse School of Graduate Studies

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Four populations of nine-banded armadillos, *Dasypus novemcinctus*, were investigated in the south-central United States in order to assess genetic variation in an isolated population (Electric Island, Lake Hamilton, Garland County, Arkansas); a semi-isolated population (Arkansas Post, Arkansas County, Arkansas), and two free ranging populations (southern Arkansas and central Texas). A 233 basepair sequence of the D-loop region of mitochondrial DNA was sequenced in individuals from each population. Individuals and populations were compared to assess relatedness among populations and individuals. Higher sequence diversity was detected in the semi-isolated population, while lower sequence diversity was observed in the isolated and free ranging populations. Overall, all populations exhibited low genetic variation when compared to genetic variation for other mammals. The results support the hypothesis that rapid range expansion combined with the organism's unique reproductive strategies have promulgated low genetic variation in the North American populations of nine-banded armadillos.

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INTRODUCTION

The appearance of powerful molecular tools for revealing genetic variation has greatly facilitated the understanding of populations, population sub-divisions and its relation to geographic barriers, and the nature and extent of gene flow (Zink, *et al.*, 1991). Geographical boundaries and behavioral patterns are believed to cause the phylogeographic structuring of species (Avise, *et al.*, 1987). Genetic distance can be correlated with geographical distance if barriers do not limit the movement of conspecifics. However, if a terrestrial species is distributed on islands, mountaintops, or other isolated areas, they may exhibit genetic differentiation due to reproductive isolation (Hoelzel and Dover, 1991).

Mitochondrial DNA Characteristics

Vertebrate mtDNA is conservative in size, function and organization. Sizes of the molecules range from 16.7 kilobases (kb) to 19.5 kb in multi-cellular animals. The molecule has been sequenced and sized for many vertebrates including mice and humans (*Mus,* 16295 basepairs, bp; *Homo,* 16569 bp; Brown, 1983). Animal mtDNA genetic code differs from the standard universal code in that ATA codes for methionine, TGA codes for tryptophan, and AGR codes for the termination of transcription (Hartl and Clark, 1989).

Animal mtDNA is a double-stranded, covalently closed circular molecule containing 2 ribosomal (rRNA) genes, 22 transfer RNA (tRNA) genes, and 13 protein genes, which code for subunits of enzymes in electron transport or ATP synthesis. The G+C content of the molecule ranges from 37-50% in vertebrates (Brown, 1983). Both

replication and transcription occur within the organelle, autonomous of nuclear DNA (Avise and Lansman, 1983). Mitochondrial DNA is functionally different from nuclear DNA in a number of ways. Replication of animal mtDNA is both unidirectional and asymmetrical, in that one of the strands, the heavy strand (H strand) contains the majority or transcribed regions. Of the 37 transcribed animal mtDNA genes, only nine (eight tRNAs and one mRNA) are on the light strand (Brown, 1983).

Mitochondrial DNA is maternally inherited through the egg cytoplasm with few exceptions (Hoelzel and Dover, 1991). The midpiece of mature sperm carries approximately 50-100 mtDNA molecules and in some instances these molecules may disperse into the zygote cytoplasm during fertilization. However, the preponderance of egg mtDNA in zygotes can account for the common observation that most mtDNA is maternally inherited (Avise and Lansman, 1983).

The issue of maternal inheritance and haploidy of mtDNA are central to its use in population genetics. There are approximately 10^5 mitochondria in a mammalian egg and approximately 50 in the midpiece of a mammalian sperm. If the sperm contributes no mitochondria to the subsequent generation, and the mtDNA in the egg is homogeneous, then the mtDNA will be transmitted as a haploid genome, and only within a few matrilines. This makes mtDNA a powerful marker for lineage studies.

Ninety percent of the mtDNA genome is transcribed. Intervening sequences (introns) have been shown to be completely absent from the sequenced mtDNAs of several vertebrates, including humans (*Homo sapiens*, Anderson, *et al.*, 1981), bovine (*Bos taurus,* Anderson, *et al*., 1982), and mice (*Mus musculus,* Bibb, *et al.*, 1981). In

vertebrates, most structural genes are separated instead by tRNAs, which are thought to act as signals for processing of the transcript. A non-coding region (or displacement loop, D-loop) region is also present, which lacks structural genes but contains sequences that initiate replication of the H-strands and also contains highly variable non-transcribed regions (Hoelzel and Dover, 1991; Moritz *et al.*, 1987)

Mitochondrial DNA D-loop

In vertebrates, the control region contains a displacement loop (D-loop) structure that functions in replication of the H-strand. This structure is called the displacement loop because the two parent strands are displaced by a short replication product, thus forming a loop structure (Wilkinson and Chapman, 1991). This short strand of DNA serves as a primer for heavy strand synthesis and is a major regulatory portion of many animal mitochondrial genomes (Perna and Kochner, 1996). The control region of mammalian mtDNA contains the origin of the H-strand replication between the genes for proline tRNA and phenylalanine tRNA. In mammalian mtDNA, transcription is initiated within the control region and proceeds to the end of the rRNA genes or for the full length of the coding sequences (Moritz, *et al*., 1987). The D-loop has been investigated extensively for comparisons of populations because it is non-coding, highly variable, and matrilineally inherited (Hoelzel and Dover, 1991)

Evolution Rates for mtDNA

The rate of mtDNA sequence evolution, relative to that of nuclear DNA varies between groups of organisms, but has been shown to evolve at five to ten times the rate of DNA of primates (Moritz, *et al*., 1987). Molecular evolution of the mitochondrial

genome in rodents has been estimated to be approximately 1.5-2 times faster that other mammalian lineages (Arborgast, 1999). Among vertebrate mtDNAs, transitions (A↔G, C↔T) far outnumber transversions (A↔C, A↔T, G↔C, G↔T). This higher frequency of transitions is observed at all codon positions, as well as in tRNA and rRNA sequences (Moritz, *et al*., 1987). Transversions occur at a much lower rate and therefore will take longer to accumulate in a population (Brown, 1983). One theory that addresses these rapid mtDNA evolutionary rates centers on the lack of evidence of a DNA repair mechanism in animal mtDNA. Mammalian mtDNA is replicated by γ-polymerase, an enzyme that lacks a proofreading function, and is prone to a higher rate of nucleotide misincorporation than nuclear DNA polymerases (α-polymerase) (Brown, 1983).

The D-loop region of the animal mitochondrial genome has been shown as the most polymorphic in the highly variable molecule. The substitution rate in the human D-loop region is estimated to be 2.8 –5 times that of the rest of the mitochondrial genome (Hoelzel and Dover, 1991)

Population genetic theory suggests that mtDNA should be a sensitive indicator of female-mediated gene flow, founder events, and other populational level processes (Brown, 1983). Mitochondrial DNA markers are expected to show greater differences than nuclear markers between demes, especially where females are more sedentary than males.

Analysis of mtDNA has also been used to date events in the history of a species, on the assumption that mtDNA diversity within a species is correlated with the time since a maternal ancestor was last shared. The rate of mitochondrial DNA evolution has been

studied thoroughly in many organisms; nucleotide differences have been shown to accrue at approximately 0.5% to 1% per million years per lineage in humans (Moritz, *et al*., 1987). Since this association between mtDNA sequence divergence and approximate divergence time was established for higher primates, a mtDNA "clock" has been widely employed to estimate divergence time within and among species. Mitochondrial DNA thus represents a powerful fusion between molecular and evolutionary biology (Moritz, *et al*., 1987).

Mitochondrial DNA and Population Genetics

DNA amplification by PCR (Mullis and Faloona, 1987) offers a method for obtaining genetic material from populations with relative ease. This portion of the matrilineally-transmitted mitochondrial genome that has been used extensively for population comparisons and has been used successfully to discern historical events such as genetic bottlenecks as well as contemporary population structure in a number of studies (*e.g*. Rosen and Block, 1996; Toline and Baker, 1995; and Fajen and Breden, 1992). Because mtDNA variation is distributed primarily among, rather than within, populations of non-human animals (Avise, *et al.*, 1987; McKnight, 1995; Moritz, *et al.*, 1992), a large number of organisms per population are not needed.

D-loop variation can be found in two forms: length variation and nucleotide sequence variation. Length variation can occur when tandem repeats or single base insertions/deletions occur in or around the control region. The continuation of any new mutation needs only an individual with multiple forms of the mitochondrial genome coexisting within that individual (Brown, *et al.*, 1996). When an individual is found to

have more than one mtDNA form, then it is said to be heteroplasmic. Point mutation heteroplasmies were reported to be rare (Moritz, *et al.*, 1987); however, recent studies utilizing newer testing methods such as denaturing gradient gel electrophoresis (DGGE), have demonstrated that more heteroplasmies, as well as single nucleotide polymorphisms, exist than originally thought. It has been suggested that since heteroplasmy is rare among mammals, endotherms, with higher metabolic rates, may experience stronger selection for smaller and less variable mtDNAs than ectotherms (*e.g*., Rand and Harrison, 1989).

Length variation heteroplasmies have been reported frequently (Rand, 1993). The most commonly reported vertebrate form of length variation heteroplasmy consists of tandemly repeated bases, such as poly-C or poly-A stretches in the D-loop or control region (Brown, *et al.*, 1996). Nucleotide sequence variability caused by point mutations and length variation has been recorded within and between many species (*e.g*. trout, *Salmo trutta*, Apostolidis, *et al.*, 1997; sheep, *Ovis canadensis*, Boyce, *et al.*, 1999; tiger salamander, *Ambystoma tigrinum*, Shaffer and McKnight, 1996; and domestic dog, *Canis familiaris,* Vila, *et al.*, 1999). Nagata, *et al*. (1998) reported that the number of tandem repeats could be a useful genetic marker for population identification.

Variation in nucleotide sequence in the D-loop has been recorded both within and between species. It has been suggested that this variation is evidence for co-evolution of nuclear-mitochondrial genomes and is based on the relationship between mitochondrial DNA and nuclear DNA regarding the coding of polymerases for both replication and transcription. This assumes that mtDNA D-loop sequence has been under selection pressure to assure nuclear enzyme binding (Wilkinson and Chapman, 1991). Alternately,

but not exclusively, it has been suggested that the mtDNA species specificity may be the result of concerted evolution, *i.e.* tandemly repeated sequences through replication slippage and unequal crossing over (Hillis, *et al.*, 1993; Wilkinson and Chapman, 1991). These D-loop sequences could be generated through concerted evolution regardless of the functional relationships (replication and transcription) if the substitutions were accumulated independently through unrelated lineages (Wilkinson and Chapman, 1991).

Study Animal

Natural History

The nine-banded armadillo or common long-nosed armadillo (*Dasypus novemcinctus mexicanus*) is the only member of the order Xenarthra found in North America. This animal is a small to medium-sized, burrowing, insectivorous mammal with an average weight of 4.0-8.0 kg and length of 70.0-100.0 cm. Nine-banded armadillos are solitary and somewhat philopatric with stable home ranges averaging from 2.5-ha (Fitch *et al.*, 1952) to 4.0-ha (Schell, 1994). The preferred habitat includes woodlands, savannah (Jones *et al.*, 1983; Layne and Glover, 1985), and bottomland riparian zones to brushy, forested upland areas (Fitch, *et al.*, 1952; Zimmerman, 1990). Forest leaf litter (10.0-12.0 cm deep) is the preferred foraging area where the armadillos detect invertebrates by smell (Talmage and Buchanan, 1954). Audubon and Bachman first reported the presence of nine-banded armadillos in North America in extreme southern Texas in 1854. Since that time, they have been described as one of the most active mammalian invaders in recent history (Humphrey, 1974). Recently, Taulman and Robbins (1996) redefined the distribution of the nine-banded armadillo. Presently, the

range extends from Texas north to Kansas and east to South Carolina (Fig. 1). Reports of specimens from southern Nebraska were not considered to be parts of permanent populations. The same is thought for animals found in both the southern counties of Illinois and Kentucky. In the east, armadillo road kill have been reported as far north as North Carolina (Carroll, pers. comm.). These are also not considered to be a part of permanent populations.

Large bodies of water (Mississippi and Tennessee Rivers) have been considered to be boundaries to the nine-banded armadillos' recent range expansion into Illinois and Kentucky. The Rio Grande may also have impeded range expansion north and east prior to 1845. However, the settlement of south Texas by Europeans and their methods of land use (agricultural practices) may have facilitated the organism's movement into the United States. Many have reported that armadillos are able swimmers (Kalmbach, 1943; Taber, 1945; Talmage and Buchanan, 1954), but do not cross large bodies of water.

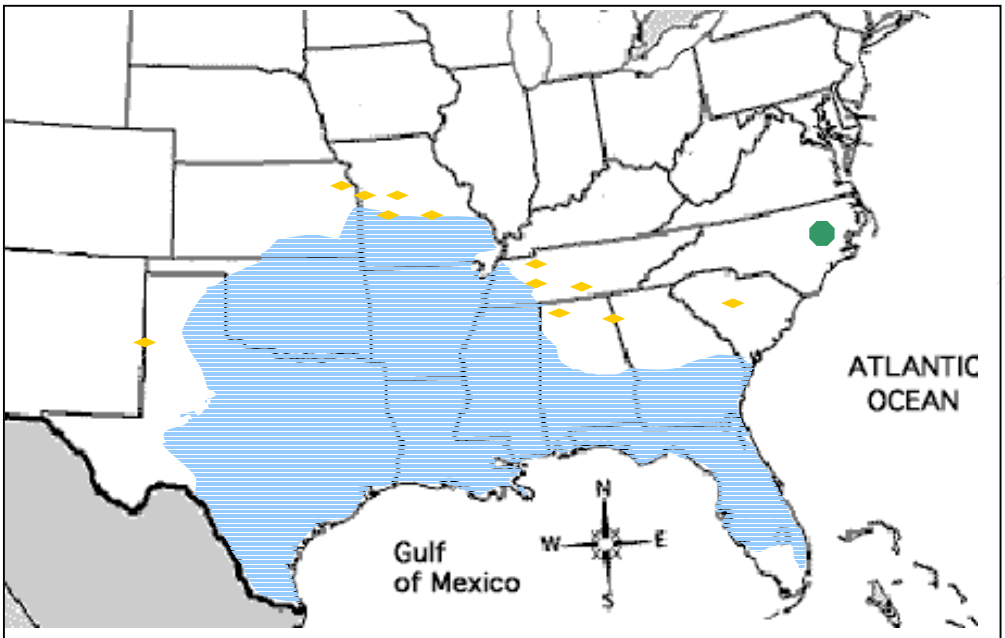
Taulman and Robbins (1996) reported that populations of armadillos have become established in areas of the Great Plains where there are from 20 to 24 total annual freeze days or mean January temperatures between 0.0 to –2.0^{o}C occur. McNab (1980) and Humphrey (1974) reported that $\leq$ nine consecutive freeze nights may limit dispersal.

Biogeography

The distribution of a population or species and genetic structure depends not only on biological and environmental conditions but also on historical factors. The spatial distribution of populations would be problematical to explain without taking all of these factors into account (Agnese, *et al.*, 1997). Taulman and Robbins (1996) reported that

the nine-banded armadillo is a recent addition to the fauna of Texas. *Dasypus bellus*, a larger armadillo (20-30 kg) similar to *D. novemcinctus,* resided in North America during the Pleistocene. Fossil records indicate the range of D. *bellus* extended as far north as Iowa and Nebraska (Voorhies, 1987). McNab (1980) reported that it is unclear whether *D. novemcinctus* evolved from *D. bellus* or simply replaced it. Based on fossil records, it has been hypothesized that neotropical populations are the source for the current distribution of the nine-banded armadillo (Edmund, 1985).

Fig. 1.—North American distribution of nine-banded armadillos, *Dasypus novemcinctus.*



Range (Taulman and Robbins, 1996)

Individual Sightings after 1994 (Taulman and Robbins, 1996)

Individual sightings in 2000 (Carroll, pers. comm.)

Genetic Variation

Low levels of genetic variation have been previously reported for North American populations of nine-banded armadillos. Ramsey and Grigsby (1985) reported estimated overall heterozygosity values of 1.0% for armadillos across the range of the species (about 9.0% is average for animal populations) based on allozyme analysis of 28 loci (N=235). They suggested the lack of variation was possibly due to inbreeding and populations in Florida introduced by humans. Additionally, Moncrief (1988) suggested inbreeding reinforced by reproductive strategy were responsible for the absence of genetic variation based on allozyme electrophoresis for a population of nine-banded armadillos in Texas. Huchon, *et al.* (1999) reported low genetic variability in nine individuals of *D. novemcinctus* from Texas, Mississippi, and Louisiana and suggested this very low level of sequence diversity was a result of the founder effect.

Reproduction

Armadillos in the genus *Dasypus* exhibit monozygotic polyembryony, a reproductive strategy that is unique to this genus within vertebrates and rare in the animal world (Gleeson, *et al*., 1994; Hardy 1995). A single fertilized egg gives rise to multiple embryos, following division of the inner cell mass of the blastocyst (Talmage and Buchanon, 1954). The nine-banded armadillo usually gives birth to four young, but litters of three and five have been reported (Galbreath, 1985). Other species of *Dasypus* have been reported to have from two to 12 young per litter (Table 1).

Galbreath (1985) and Peppler (1976) reported that ovulation for nine-banded armadillos in Florida and Texas occur most often in summer, implantation in fall (3.5

month delay), and birth in March and April (4.5 month gestation). Delayed implantation is a reproductive tactic utilized by many mammals from several orders (e.g. Artiodactyla, Carnivora, Chiroptera). In most mammals, implantation will occur in the late winter or early spring when resources again become plentiful. It has been suggested that this reproductive strategy evolved to ensure mating and parturition occur when temperature and food conditions are optimal for the young's survival.

The kite-shaped uterus of the nine-banded armadillo is unique in that it has a large fundic cavity, endometrial grooves, and a fundic apical depression. This depression is the location of the blastocyst's delay site and is also the location of implantation. The endometrial grooves appear to guide the blastocyst toward the fundic tip (Buchanon, 1967). Characteristics of the uteri of other *Dasypus* sp. have not been reported.

Table 1.--*Dasypus* sp. range, number of young, and presence or absence of polyembryony.

SPECIES	RANGE	NUMBER OF EMBRYOS REPORTED	POLYEMBRYONY DOCUMENTED
Dasypus novemcinctus, the nine-banded armadillo	Southern United States to Mexico, Central America, and South America west of the Andes and northeastern Peru and east of the Andes to Argentina	4 same sex	Talmage and Buchanon, 1954
Dasypus hybridus, the southern lesser long-nosed armadillo	Northern Argentina and central and southern Paraguay south east through Uruguay and Southern Brazil and extreme east central Argentina	≤12 same sex	Galbreath, 1985
Dasypus sabicola, the Northern lesser long-nosed armadillo	Savannas of Venezuela and Columbia	4 same sex	Galbreath, 1985 No
Dasypus kappleri, the greater long nosed armadillo	Forested areas of Columbia, east of the Andes into Guyana, Suriname, and French Guiana through most of the Amazon Basin	2 same sex	Galbreath, 1985 No
Dasypus septemcinctus, lesser long-nosed armadillo	Mouth of the Amazon south through highlands of Brazil and into Bolivia and Argentina	?	No
Dasypus pilosus, hairy long nosed armadillo	mountains of Peru	?	No

OBJECTIVES

The objectives of this study were as follows: 1) to determine the genetic effects of population subdivision due to a vicariant isolation event (reservoir impoundment) in a population of nine-banded armadillos on Electric Island; 2) to determine the genetic effects of a filter barrier on a population of nine-banded armadillos on Arkansas Post; 3) to assess whether genetic variation has been affected or significantly reduced in the isolated and semi-isolated population; 4) to examine two free ranging populations of nine-banded armadillos--one from Arkansas and the other from Texas--to determine the amount of genetic variation within and between each population; 5) to compare the isolated and semi-isolated populations to the unrestricted populations in regard to genetic variation; and 6) assess reproductive strategies and levels of genetic variation in regard to rapid range expansion in nine-banded armadillos.

MATERIALS AND METHODS

Tissue Collection

Nine-banded armadillos were collected by live trapping from September 1994 until July 1996 (Table 2). Armadillos from all localities (FRT-N=7, FRA-N=7, AP-N=4, EI-N=5) were sexed, examined for pregnancy, and ear clipped for tissue samples to be used in DNA testing. When pregnant individuals were captured, they were sacrificed, the fetuses removed, and tissues from the related specimens were collected for molecular analysis.

DNA Extraction

DNA was extracted by mincing ear tissues followed by incubation in protein lysis buffer (50mM EDTA, 20.0 mg/ml Proteinase K, 10mM Tris-HCl, 0.5% SDS) in a 65^{o}C shaking water bath for 18-24 hours. Subsequent phenol-chloroform-isoamyl alcohol (25:24:1) extractions were performed to remove proteins and lipids from the extract. Genomic DNA was precipitated with 2 volumes of cold 100% ethanol. Residual salts were removed by washing with 70% cold ethanol. Samples were air dried and resuspended in 100 ml sterile deionized water. Extraction success was evaluated through electrophoresis on a 1.0% agarose gel and staining with 0.5 ug/ml ethidium bromide for visualization. Spectrophometric readings were taken to determine DNA concentrations for each sample.

DNA Amplification

Purified genomic DNA from 27 specimens was used to amplify a 399 bp portion of the mitochondrial D-loop. Three specific primers were designed based on the

Table 2.-- Specimen localities of nine-banded armadillos, *Dasypus novemcinctus*, collected from 1994 to 1996 in Texas and Arkansas.

SPECIMEN ID	LOCALITY/OTHER	COUNTY	STATE
AP2	Arkansas Post	Arkansas	AR
AP6	Arkansas Post	Arkansas	AR
AP4	Arkansas Post	Arkansas	AR
AP10	Arkansas Post	Arkansas	AR
BG2	Electric Island	Garland	AR
BG3	Electric Island	Garland	AR
F3	Electric Island	Garland	AR
F6	Electric Island	Garland	AR
F7	Electric Island	Garland	AR
F13	Free ranging (Electric Island/Mainland)	Garland	AR
M7	Free ranging	Union	AR
M21	Free ranging	Ouachita	AR
B2	Free ranging	Little River	AR
B3	Free ranging	Little River	AR
B4	Free ranging	Little River	AR
G1	Free ranging	San Saba	TX
G2	Free ranging	San Saba	TX
G3	Free ranging	San Saba	TX
G4	Free ranging	San Saba	TX
HI	Free ranging	San Saba	TX
K8	Free ranging	San Saba	TX
K21	Free ranging	Llano	TX
I1**	Free ranging	San Saba	TX
I4*	Free ranging	San Saba	TX
I5*	Free ranging	San Saba	TX

** - mother, *offspring of I1 collected in-utero

mitochondrial DNA sequence published by Arnason, *et al.* (1997, EMBL #Y11832) for the 1604 base pair control region. The forward primer was located at 16001 to 16025 with the sequence of 5'-TCACCTAAAACCGTCCACTCATTCC-3'. Two reverse primers were located at 16403 to 16429 (=I) with sequence of 5'-GTATATAATAAATTGTGCGTATGCGTA-3' and 164625 to 16450 (=II) with the sequence of 5'-ATATGATAAAAGATAACGGTTTGGGG-3'. The second reverse primer was used in a nested PCR procedure using the amplicons produced by the initial reaction as a template in an attempt to enhance specificity for the amplicons of interest. For amplification procedure I, 10-15 ng of each DNA sample was amplified using Perkin Elmer's *AmpliTaq Gold™DNA Polymerase* (one unit per reaction) and *GeneAmp™ PCR Buffer II*, and 10 millimolar concentrations of the forward primer and reverse primer II, respectively. Amplification was performed with a Perkin Elmer Model 9600 thermocycler set at the following parameters: 95°C hold for 11 minutes, then 35 cycles of the following: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds; followed by a 72°C hold for 3 minutes, ending with a 15°C indefinite hold. PCR products were then electrophoresed on a 2.0% agarose gel stained with *SYBR® Gold* solution for visualization, then the gel was scanned and images were produced to confirm the amplification of the desired fragment. The PCR products were amplified again following the same parameters as above, but with the following exceptions: reverse primer I was substituted for reverse primer II and the annealing temperature was changed from 50°C to 52°C. Unincorporated nucleotides, polymerases, and primers were removed from PCR products with *Microcon 100™* concentrators.

DNA Sequencing

Purified amplicons were sequenced with the ABI Prism^{TM} dRhodamine terminator Cycle Sequencing Reaction Kit. The reaction was carried out by combining kit reagents, mtDNA template, and the internal primer (5'-CACCGACTCACCTATGCC-3') in amplification tubes and using the Perkin Elmer 9600 thermocycler. The amount of template used for these reactions was between 10-35 ng in 5 ul of TE buffer. The thermocycler parameters were as follows for the forward sequence: 95^{o}C hold for 1 minute, then 25 cycles of the following: 96^{o}C for 16 seconds, 50^{o}C for 1 seconds, 60^{o}C for 1 minute; followed by a 15^{o}C hold for 10 minutes. For each sample, 5 ul template was mixed with 2.4 ul of each primer (F and RPI) (10mM), 4 ul Sequencing Ready Reaction Mix, and 0.6 ul sterile H_2O for a total reaction volume of 12 ul. With each cycle sequencing reaction, a control template (pGEM-3Zf) was also included. The pGEM reaction volumes were as follows: 3 ul templates, 1.5 ul primer, 9.5 ul Sequencing Ready Reaction Mix, and 6 ul H_2O. Following the cycle sequencing reaction, the extension products were ethanol precipitated with 70% ethanol/0.5 mM $MgCl_2$ solution. For each sample, 75 ul of the preceding solution was added, and incubated at room temperature for 15 minutes to precipitate the extension produced. The tubes were then centrifuged for 30 minutes at 5.5 rpm, opened and inverted on a paper towel to remove the ethanol solution. Tubes were placed open and inverted into 1.5 ml microcentrifuge tubes and centrifuged again for 1 minute at 2 rpm.

After sequencing reactions were completed, each sample ethanol precipitated to purify the extension product, then resuspended in 25 ul Template Suppression Reagent

(Perkin Elmer). The samples were vortexed and centrifuged for collection. The samples were heated at 95°C for 2 minutes to denature the extension products. Samples were again vortexed, and then loaded for running on an *ABI PRISM 310 Analyzer*.

Statistical Analysis

Arlequin (Schneider, *et al.*, 2000) was used to analyze the relationships among haplotypes identified by mtDNA sequencing and MEGA, version 1.01 (Molecular Evolutionary Genetic Analysis, Kumar, *et al.*, 1993) was used generate dendrograms of the individual relationships. Tree-building procedures using the entire data set included neighbor joining and branch swapping. Distance estimates were assessed using the Kimura two parameter model (Kimura, 1980).

Study Sites

This study was conducted with specimens from populations from four areas of the south-central United States [central Texas (free ranging); south Arkansas (free ranging); central Arkansas (Electric Island, Lake Hamilton); and eastern Arkansas (Arkansas Post, Arkansas County)].

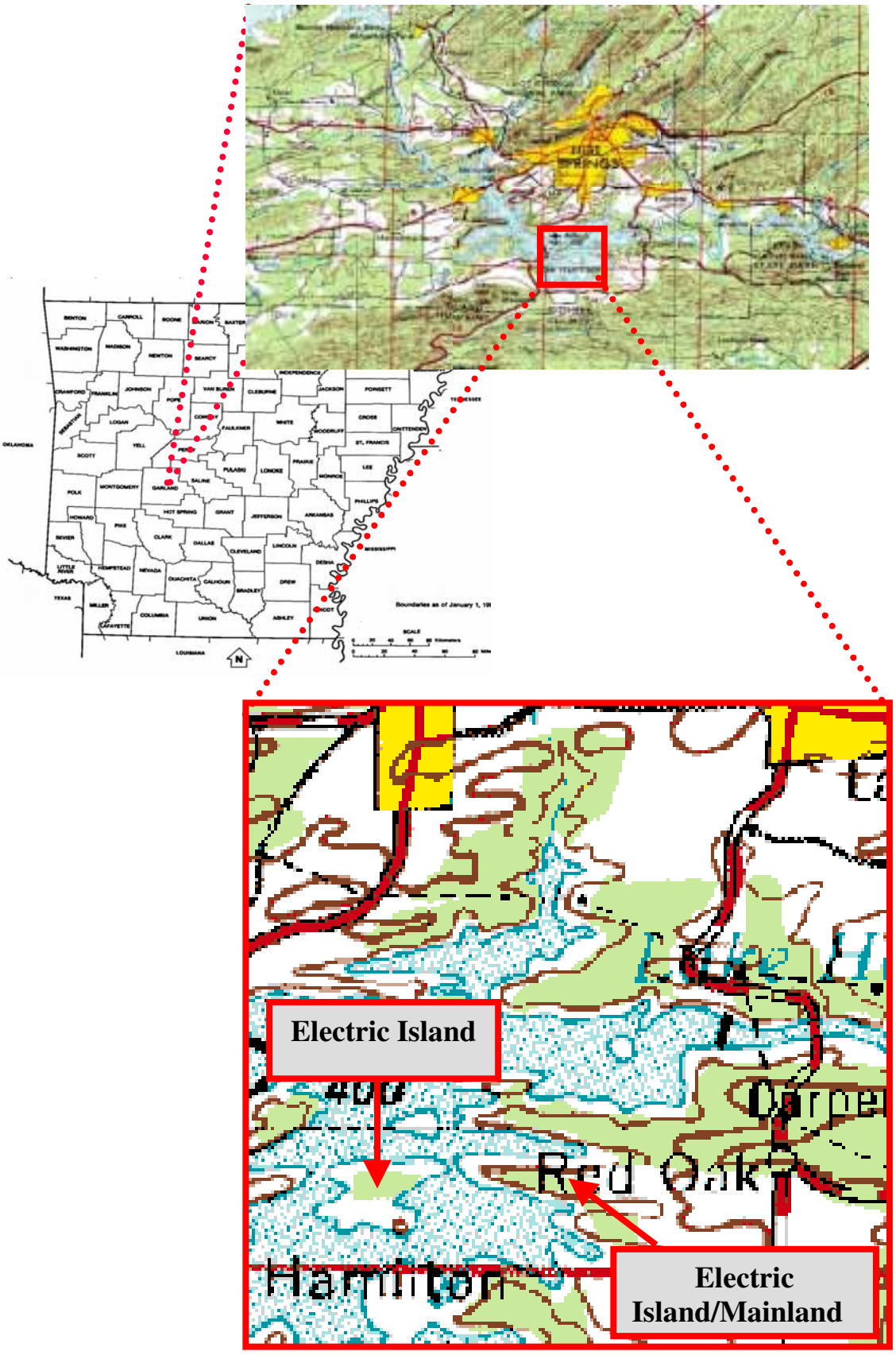
Electric Island

In the late 1920s and early 1930s, the Arkansas Power and Light Company impounded the Ouachita River (Garland County, Arkansas). This area is located in the Ouachita Mountains and due to the impoundment, two lakes were formed (Lake Hamilton and Lake Catherine) and several areas of higher elevation were isolated as islands within these lakes. In the 1950s, a third lake, Lake Ouachita, was created immediately to the northeast. Lake Hamilton, the largest of the two lakes, is a 3,270 ha

reservoir.

Within Lake Hamilton many islands are located, with the largest being Electric Island (Figure 2). It is a 47 ha island owned by the Arkansas Nature Conservancy and managed by the Arkansas Game and Fish Commission. The island and the nearby mainland are composed of oak-hickory-pine-forest with abundant leaf litter and snags (Smith, *et al.*, 1983). Soils are composed mainly of poorly drained, shallow sandy loams interspersed with shale and sandstone as primary surface rocks. Electric Island is located approximately 300 m from the closest point of the mainland. Annual winter drawdowns of one to two meters begin in November and end in mid-March. Water levels decrease such that the underwater distance from the island to the mainland decreases to approximately 150 m.

Fig. 2.--Electric Island, Lake Hamilton, Garland County, Arkansas.


Electric Island
Red Oak
Hamilton
Electric Island/Mainland

Arkansas Post

Located in Arkansas County in southeastern Arkansas, Arkansas Post (Fig. 3) is a 157.4 ha peninsula, bounded by the Arkansas River to the south, and backwaters of Post Bend to the east, and Post Bayou, Moore Bayou, and Little Post Bayou to the west. It is located in the delta region of the state that is characterized by lowland hardwood forest mixed with riparian areas and farmland. A 2.8 ha lake is located in the south central portion of the peninsula. The entrance from the mainland is located on the northwestern side of the peninsula. Maximum bayou depth is 1.2 m and the minimum distance from the mainland (excluding bridge) is $\leq$ 5.0 m. Soils consist of sandy loam.

In 1686, the French claimed the area and named it Poste de Arkansas (Arkansas Post) at the Quapaw village of Osotuoy. The site is historically significant in regard to the River Road, the Revolutionary War, and the Civil War. The Post was moved seven times during its history due to the flooding of both the Arkansas and Mississippi Rivers. Arkansas Post is now a national memorial and state park.

South Arkansas Free Ranging Populations

Specimens were collected from four counties in southern Arkansas: Miller, Ouachita, Ashley, and Little River counties (Fig. 4). All of these counties are located in the West Gulf Coastal Plain region of the state. This area is characterized by mixed pine and hardwood forests interspersed with farmland. Many river systems traverse this area such as the Ouachita River, Saline River, and Little River. Soils consist of well-drained, deep sandy, or silty clay loams (Selander and Heidt, 1990).

Fig. 3.--Arkansas Post, Arkansas County, Arkansas.

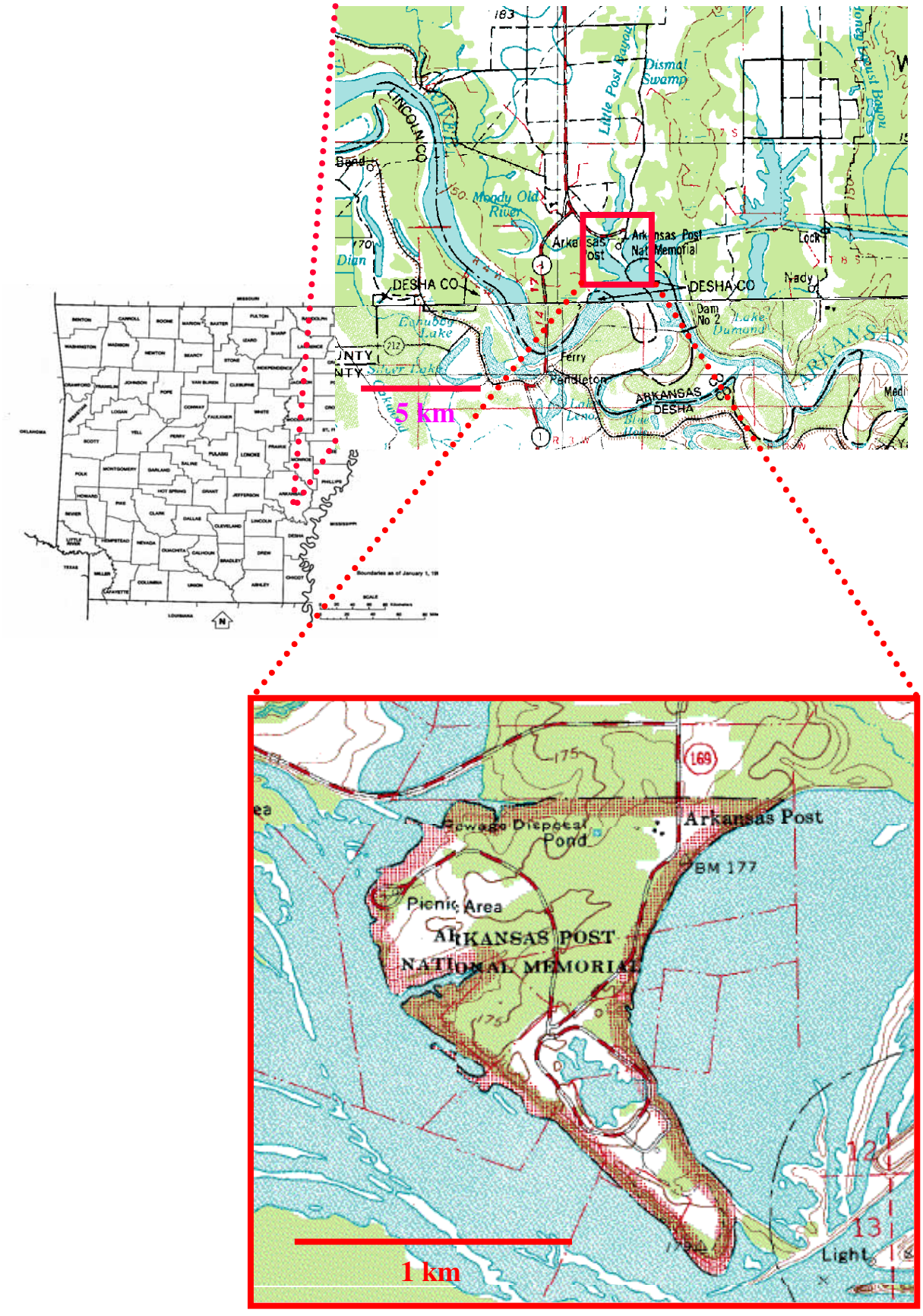
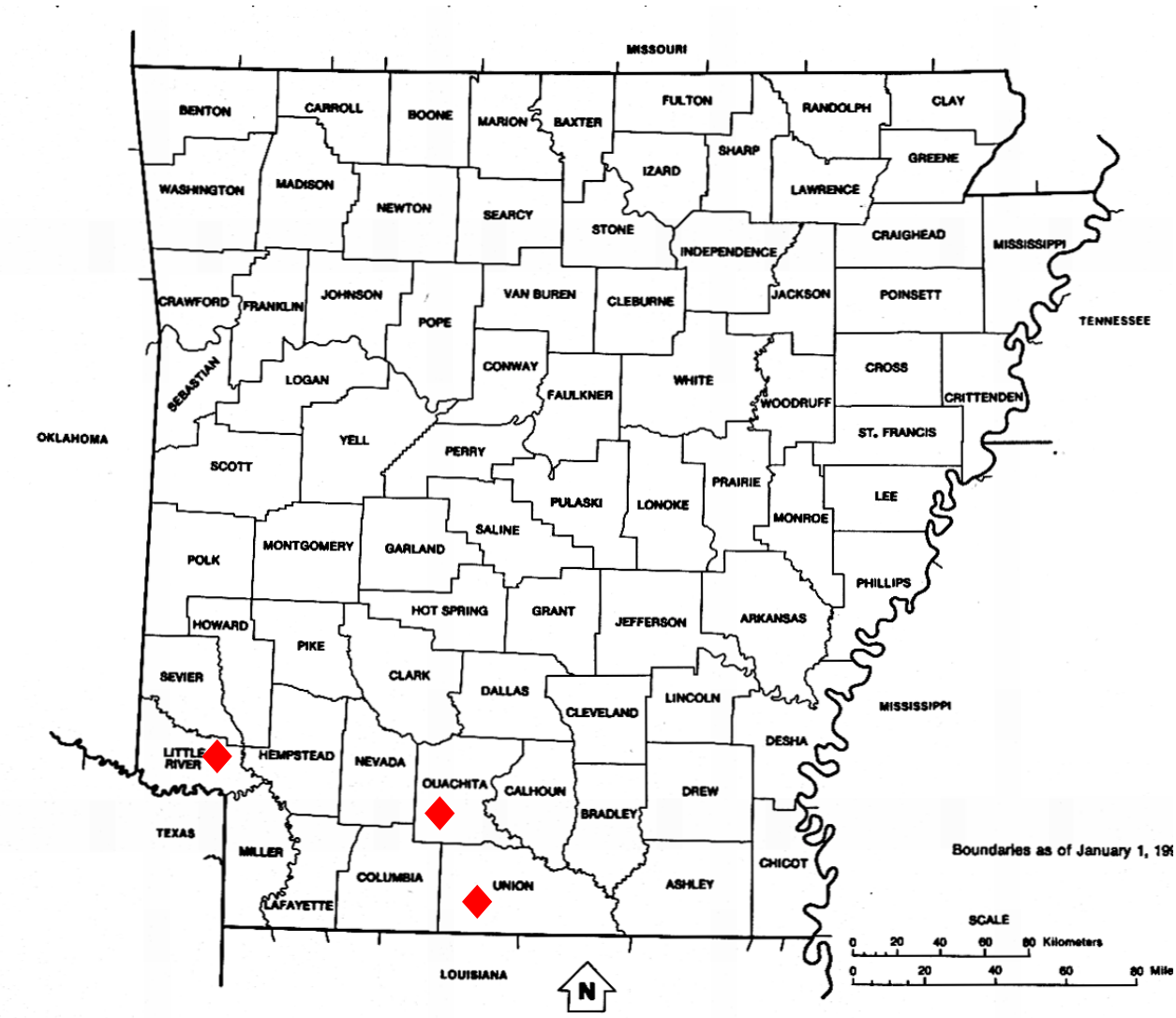

Dismal Swamp
Little Post Bayou
LINCOLN CO
Bend
Moody Old River
Arkansas Post Nat Memorial
DESHA CO
Lock
Nady
Dam No 2
Lake Dumond
Ferry
Pendleton
ARKANSAS
DESHA
5 km
Arkansas Post
BM 177
Picnic Area
ARKANSAS POST
NATIONAL MEMORIAL
169
175
12
13
Light
1 km

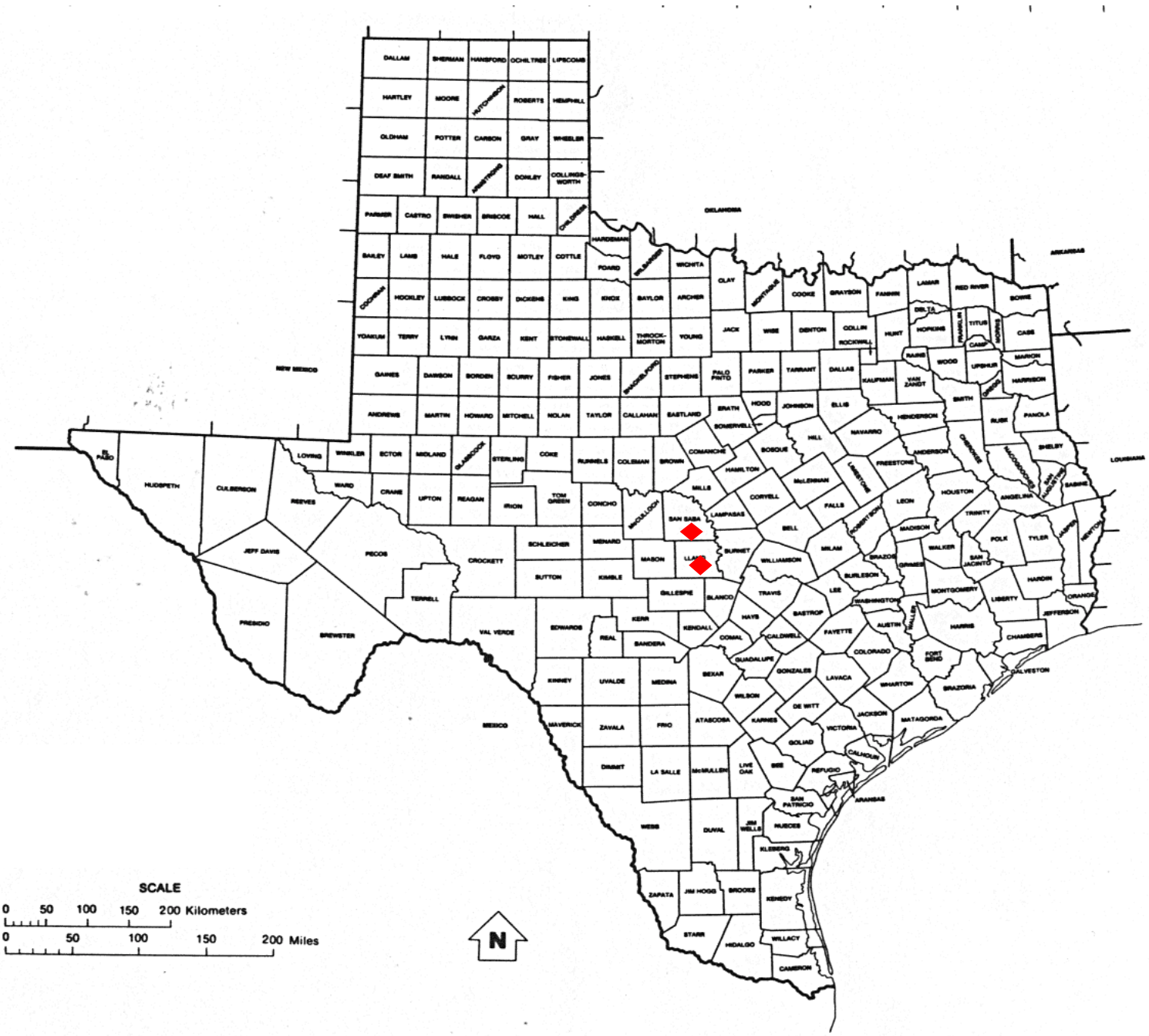
Fig. 4.--Localities at which nine-banded armadillos, *Dasypus novemcinctus,* (diamonds) were collected in southern Arkansas, 1994-1996.


MISSOURI
BENTON
CARROLL
BOONE
MARION
BAXTER
FULTON
RANDOLPH
CLAY
WASHINGTON
MADISON
NEWTON
SEARCY
IZARD
SHARP
GREENE
LAWRENCE
STONE
INDEPENDENCE
CRAIGHEAD
MISSISSIPPI
CRAWFORD
FRANKLIN
JOHNSON
POPE
VAN BUREN
CLEBURNE
JACKSON
POINSETT
TENNESSEE
SEBASTIAN
LOGAN
CONWAY
FAULKNER
WHITE
WOODRUFF
CROSS
CRITTENDEN
OKLAHOMA
YELL
PERRY
ST. FRANCIS
SCOTT
PULASKI
LONOKE
PRAIRIE
LEE
SALINE
MONROE
POLK
MONTGOMERY
GARLAND
PHILLIPS
HOT SPRING
GRANT
JEFFERSON
ARKANSAS
HOWARD
PIKE
SEVIER
CLARK
DALLAS
CLEVELAND
LINCOLN
MISSISSIPPI
DESHA
LITTLE RIVER
HEMPSTEAD
NEVADA
OUACHITA
CALHOUN
BRADLEY
DREW
TEXAS
MILLER
COLUMBIA
UNION
ASHLEY
CHICOT
LAFAYETTE
LOUISIANA
N
Boundaries as of January 1, 19
SCALE
0 20 40 60 80 Kilometers
0 20 40 60 80 Mile

Central Texas Free Ranging Populations

Specimens were collected from two counties in central Texas: San Saba and Llano counties (Figure 5). Both are in the Llano Uplift, which occurs within the Edwards Plateau (Davis and Schmidly, 1994). The vegetation consists mainly of oak and oak-hickory, mesquite savanna and some grassland. Sandy soils predominate in this region.

Fig. 5.—Localities at which nine-banded armadillos, *Dasypus novemcinctus,* (diamonds) were collected in central Texas, 1994-1996.


SCALE
0 50 100 150 200 Kilometers
0 50 100 150 200 Miles
N
SAN SABA
LLANO
OKLAHOMA
NEW MEXICO
MEXICO
ARKANSAS
LOUISIANA

RESULTS

Nine haplotypes were observed among the four populations (N=24) of nine-banded armadillo mtDNA sequenced (Table 3 and Table 4). Within the 233 bp region of the D-loop, 13 sites were polymorphic and four were phylogenetically informative (Table 5). Of the polymorphisms detected, nine were transitions, five were transversions, 22 were deletions, and there was one observed insertion. The average nucleotide composition for this region for all individuals sequenced was 25.8%A, 31.2%T, 22.1%C, and 20.8%G.

Pairwise genetic distances were computed using the Kimura-2 Parameter model in MEGA (Schneider, *et al.,* 2000), which considers rates of transitional nucleotide substitutions as being higher than those of transversional substitutions—as is the case for animal mitochondrial DNA (Brown *et al*., 1983). Estimated values of mean pairwise distances (sequence divergence) between all pairs of individuals for the basepair D-loop region--calculated by the Kimura 2 parameter algorithm method--range from 0.0000 to 0.0308 (Table 6). The transition to transversion ratios ranged from 0.000 to 0.667 (Table 6). The mean number of pairwise differences overall was 0.045%.

The Electric Island individuals (N=5) displayed 3 variable characters for this region. The mean number of pairwise differences in the island population was 14.67 +/- 9.12 and the nucleotide diversity was 0.043011 +/- 0.033344. The deletion at nucleotide position 16125 was present in all individuals sampled from this location.

The Electric Island/Mainland individual displayed 2 variable characters for the 233 basepair D-loop region. The deletion at nucleotide position 16125 was present in this individual.

The Arkansas free ranging individuals (N=5) displayed seven variable characters for this region. The mean number of pairwise differences in this population was 9.33 +/- 5.93 and the nucleotide diversity was 0.032520 +/- 0.025750. The deletion at nucleotide position 16125 was absent in all individuals sampled from this location. Pairwise genetic distances among the five individuals sampled ranged from 0.0140 to 0.0400 for the 233 basepair fragment (Table 3).

The Texas free ranging individuals (N=10) displayed seven variable characters for this region. The mean number of pairwise differences in this population was 9.33 +/- 5.93 and the nucleotide diversity was 0.032520 +/- 0.025750. The deletion at nucleotide position 16125 was present in five individuals sampled from this location. An insertion was found at nucleotide position 16125.1 in one individual sampled from this location. Pairwise genetic distances among the 10 individuals sampled ranged from 0.0140 to 0.0400 for the 233 basepair fragment (Table 3).

The Kimura-2 Parameter matrix of genetic distances revealed trees with identical topologies (Figure 5) regardless clustering building method used. Two Arkansas Post individuals—AP6 and API0--consistently grouped together (group 1). The third Arkansas Post specimen (AP7) did not cluster with the other Arkansas Post individuals, but did consistently cluster with three Electric Island and one Arkansas free ranging individual using the above clustering method (group 2).

Table 3.-- Mitochondrial DNA D-loop sequence haplotype designation, descriptions, and percent distribution for nine-banded armadillos (*Dasypus novemcinctus*).

HAPLOTYPE DESIGNATION	HAPLOYTYPE DESCRIPTION	NUMBER OF SPECIMENS	%
A	Same as reference (Arnason et al., 1997)	3	12.5
B	16125-del (G)	7	29.1
C	16125.1 (G) ins	1	4.2
D	16125-del (G) 16158 T (T)	3	12.5
E	16125-del (G) 16159 T (T)	5	20.8
F	16085.1 (C) ins 16193 T (A)	1	4.2
G	16125-del (G) 16158-del (C) 16159 T (T)	2	8.3
H	16125-del (G) 16158-del (C) 16159 T (T) 16166 T (G) 16167 T (C) 16179 T (T)	1	4.2
I	16125-del (G) 16159 T(T) 16163 T (T) 16164 T (A) 16166 T (G) 16167 T (C)	1	4.2
TOTAL		24	100

Using the same distance matrix (Kimura 2 parameter), with the neighbor joining clustering method and bootstrapping analysis (to determine confidence levels at the nodes with 1000 iterations), produced a similar tree. Again, the major difference observed involved the two Arkansas Post individuals clustering in a single group, while individual AP7 still clustered with three Electric Island specimens and one Arkansas free ranging individual in a second group.

Table 4.-- Mitochondrial DNA D-loop sequence haplotypes for nine-banded armadillos, *Dasypus novemcinctus*.

SPECIMEN ID	LOCALITY	STATE	HAPLOTYPE
AP6	Arkansas Post	AR	H
AP7	Arkansas Post	AR	E
AP10	Arkansas Post	AR	I
BG2	Electric Island	AR	D
BG3	Electric Island	AR	E
F3	Electric Island	AR	B
F6	Electric Island	AR	B
F7	Electric Island	AR	E
F13	Free ranging (Electric Island/Mainland)	AR	E
M7	Free ranging	AR	C
M21	Free ranging	AR	B
B2	Free ranging	AR	E
B3	Free ranging	AR	G
B4	Free ranging	AR	D
G1	Free ranging	TX	A
G2	Free ranging	TX	A
G3	Free ranging	TX	F
G4	Free ranging	TX	A
HI	Free ranging	TX	D
K8	Free ranging	TX	G
K21	Free ranging	TX	B
I1**	Free ranging	TX	B
I4*	Free ranging	TX	B
I5*	Free ranging	TX	B

** - mother, *offspring of I1 collected in-utero

Table 5.--Nucleotide sequences for the 233 basepair region of the D-loop of 24 individuals of nine-banded armadillos, *Dasypus novemcinctus*.

```
                1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
      1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
BG2   GGACTTATGC CATATTAAAC CGTGACCTTG CATCCCCTT- ATCTGTCATA CATTTGGTAC CTTTTTTTTT
BG3   .......... .......... .......... .......... .......... .......... ..........
F3    .......... .......... .......... .......... .......... .......... ..........
F6    .......... .......... .......... .......... .......... .......... ..........
AP7   .......... .......... .......... .......... .......... .......... ..........
AP6   .......... .......... .......... .......... .......... .......... ..........
B2    .......... .......... .......... .......... .......... .......... ..........
B3    .......... .......... .......... .......... .......... .......... ..........
AP10  .......... .......... .......... .......... .......... .......... ..........
K8    .......... .......... .......... .......... .......... .......... ..........
F7    .......... .......... .......... .......... .......... .......... ..........
H1    .......... .......... .......... .......... .......... .......... ..........
K21   .......... .......... .......... .......... .......... .......... ..........
I1    .......... .......... .......... .......... .......... .......... ..........
I1    .......... .......... .......... .......... .......... .......... ..........
I4    .......... .......... .......... .......... .......... .......... ..........
I5    .......... .......... .......... .......... .......... .......... ..........
M21   .......... .......... .......... .......... .......... .......... ..........
F13   .......... .......... .......... .......... .......... .......... ..........
M7    .......... .......... .......... .......... .......... .......... ..........
G1    .......... .......... .......... .......... .......... .......... ..........
G2    .......... .......... .......... .......... .......... .......... ..........
G3    .......... .......... .......... .........C .......... .......... ..........
G4    .......... .......... .......... .......... .......... .......... ..........
```

Table 5 continued.

```
                                                1 1111111111 1111111111 1111111111 1111111111
     7777777778 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
BG2  GGGGGGGGG- -AAAAGGTCT CGACGCAGTC AATTAAATTG TAGCTGGACT TCGAATGCAC GTGATTTACC
BG3  .......... .......CT. .......... .......... .......... .......... ..........
F3   .......... .......C.. .......... .......... .......... .......... ..........
F6   .......... .......C.. .......... .......... .......... .......... ..........
AP7  .......... .......CT. .......... .......... .......... .......... ..........
AP6  .......... .......-T. .....GC... ........G. .......... .......... ..........
B2   .......... .......CT. .......... .......... .......... .......... ..........
B3   .......... .......-T. .......... .......... .......... .......... ..........
B4   .......... .......... .......... .......... .......... .......... ..........
AP10 .......... .......CT. ..TA.GC... .......... .......... .......... ..........
K8   .......... .......-T. .......... .......... .......... .......... ..........
F7   .......... .......CT. .......... .......... .......... .......... ..........
H1   .......... .......... .......... .......... .......... .......... ..........
K21  .......... .......C.. .......... .......... .......... .......... ..........
I1   .......... .......C.. .......... .......... .......... .......... ..........
I4   .......... .......C.. .......... .......... .......... .......... ..........
I5   .......... .......C.. .......... .......... .......... .......... ..........
M21  .......... .......C.. .......... .......... .......... .......... ..........
F13  .......... .......CT. .......... .......... .......... .......... ..........
M7   .........G G......C.. .......... .......... .......... .......... ..........
G1   .........G .......C.. .......... .......... .......... .......... ..........
G2   .........G .......C.. .......... .......... .......... .......... ..........
G3   .........G .......C.. .......... .......... .......... ..A....... ..........
G4   .........G .......C.. .......... .......... .......... .......... ..........
```

Table 5 continued.

```
     1111111111 1111111111 1111111111 1111111111 1111111111 1111111112 2222222222
     4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 0000000001
     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
BG2  CGCATACATT GAGTTCATGG TATTATTCAG TCAATGGTTA CAGGACATAA AAATTTTTAC GCCTTTCGCG
BG3  .......... .......... .......... .......... .......... .......... ..........
F3   .......... .......... .......... .......... .......... .......... ..........
F6   .......... .......... .......... .......... .......... .......... ..........
AP7  .......... .......... .......... .......... .......... .......... ..........
AP6  .......... .......... .......... .......... .......... .......... ..........
B2   .......... .......... .......... .......... .......... .......... ..........
B3   .......... .......... .......... .......... .......... .......... ..........
B4   .......... .......... .......... .......... .......... .......... ..........
AP10 .......... .......... .......... .......... .......... .......... ..........
K8   .......... .......... .......... .......... .......... .......... ..........
F7   .......... .......... .......... .......... .......... .......... ..........
H1   .......... .......... .......... .......... .......... .......... ..........
K21  .......... .......... .......... .......... .......... .......... ..........
I1   .......... .......... .......... .......... .......... .......... ..........
I4   .......... .......... .......... .......... .......... .......... ..........
I5   .......... .......... .......... .......... .......... .......... ..........
M21  .......... .......... .......... .......... .......... .......... ..........
F13  .......... .......... .......... .......... .......... .......... ..........
M7   .......... .......... .......... .......... .......... .......... ..........
G1   .......... .......... .......... .......... .......... .......... ..........
G2   .......... .......... .......... .......... .......... .......... ..........
G3   .......... .......... .......... .......... .......... .......... ..........
G4   .......... .......... .......... .......... .......... .......... ..........
```

Table 5 continued.

```
      2222222222 2222222222 22222
      1111111112 2222222223 33333
      1234567890 1234567890 12345
BG2   CATACGCATA CGCATACGCA TACGC
BG3   .......... .......... .....
F3    .......... .......... .....
F6    .......... .......... .....
AP7   .......... .......... .....
AP6   .......... .......... .....
B2    .......... .......... .....
B3    .......... .......... .....
B4    .......... .......... .....
AP10  .......... .......... .....
K8    .......... .......... .....
F7    .......... .......... .....
H1    .......... .......... .....
K21   .......... .......... .....
I1    .......... .......... .....
I4    .......... .......... .....
I5    .......... .......... .....
M21   .......... .......... .....
F13   .......... .......... .....
M7    .......... .......... .....
G1    .......... .......... .....
G2    .......... .......... .....
G3    .......... .......... .....
G4    .......... .......... .....
```

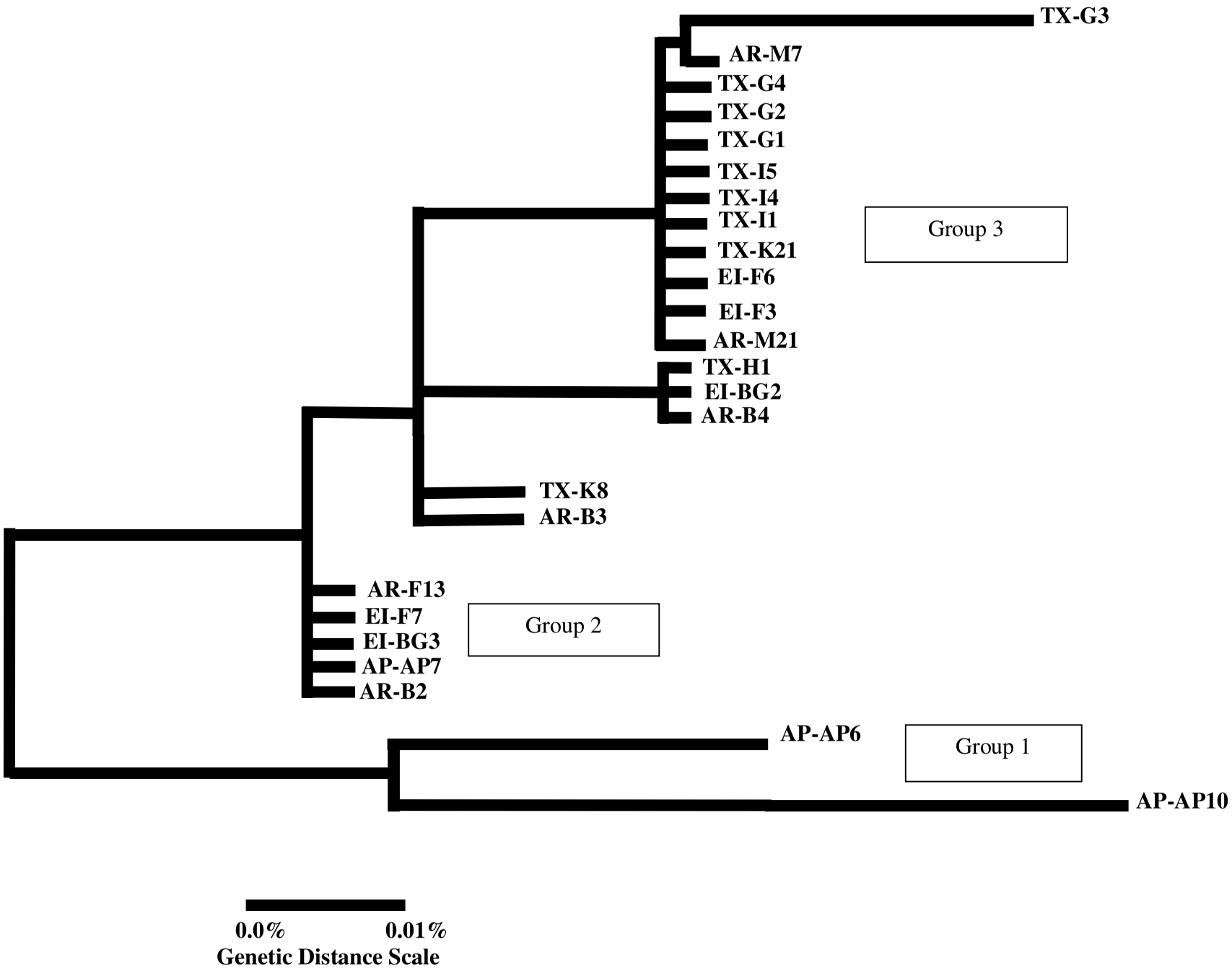
Table 6. Kimura-2 Parameter distances (above) and transition to transversion ratios (below) for the 233 basepair D-loop region of mtDNA of 24 individuals of nine-banded armadillos, *Dasypus novemcinctus*.

OTUs	BG2	BG3	F3	F6	AP7	AP6	B2	B3	B4	AP10	K8	F7	H1	K21	I1	I4
BG2		0.0087	0.0043	0.0043	0.0087	0.0175	0.0087	0.0043	0.0000	0.0263	0.0043	0.0087	0.0000	0.0043	0.0043	0.0043
BG3	2,0		0.0043	0.0043	0.0000	0.0131	0.0000	0.0000	0.0087	0.0175	0.0000	0.0000	0.0087	0.0043	0.0043	0.0043
F3	1,0	1,0		0.0000	0.0043	0.0175	0.0043	0.0043	0.0043	0.0219	0.0043	0.0043	0.0043	0.0000	0.0000	0.0000
F6	1,0	1,0	0,0		0.0043	0.0175	0.0043	0.0043	0.0043	0.0219	0.0043	0.0043	0.0043	0.0000	0.0000	0.0000
AP7	2,0	0,0	1,0	1,0		0.0131	0.0000	0.0000	0.0087	0.0175	0.0000	0.0000	0.0087	0.0043	0.0043	0.0043
AP6	1,3	0,3	1,3	1,3	0,3		0.0131	0.0131	0.0175	0.0131	0.0131	0.0131	0.0175	0.0175	0.0175	0.0175
B2	2,0	0,0	1,0	1,0	0,0	0,3		0.0000	0.0087	0.0175	0.0000	0.0000	0.0087	0.0043	0.0043	0.0043
B3	1,0	0,0	1,0	1,0	0,0	0,3	0,0		0.0043	0.0175	0.0000	0.0000	0.0043	0.0043	0.0043	0.0043
B4	0,0	2,0	1,0	1,0	2,0	1,3	2,0	1,0		0.0263	0.0043	0.0087	0.0000	0.0043	0.0043	0.0043
AP10	2,4	0,4	1,4	1,4	0,4	0,3	0,4	0,4	2,4		0.0175	0.0175	0.0263	0.0219	0.0219	0.0219
K8	1,0	0,0	1,0	1,0	0,0	0,3	0,0	0,0	1,0	0,4		0.0000	0.0043	0.0043	0.0043	0.0043
F7	2,0	0,0	1,0	1,0	0,0	0,3	0,0	0,0	2,0	0,4	0,0		0.0087	0.0043	0.0043	0.0043
H1	0,0	2,0	1,0	1,0	2,0	1,3	2,0	1,0	0,0	2,4	1,0	2,0		0.0043	0.0043	0.0043
K21	1,0	1,0	0,0	0,0	1,0	1,3	1,0	1,0	1,0	1,4	1,0	1,0	1,0		0.0000	0.0000
I1	1,0	1,0	0,0	0,0	1,0	1,3	1,0	1,0	1,0	1,4	1,0	1,0	1,0	0,0		0.0000
I4	1,0	1,0	0,0	0,0	1,0	1,3	1,0	1,0	1,0	1,4	1,0	1,0	1,0	0,0	0,0	

Table 6 continued.

OTUs	I5	M21	F13	M7	G1	G2	G3	G4
BG2	0.0043	0.0043	0.0087	0.0043	0.0043	0.0043	0.0087	0.0043
BG3	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
F3	0.0000	0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
F6	0.0000	0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
AP7	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
AP6	0.0175	0.0175	0.0131	0.0175	0.0175	0.0175	0.0220	0.0175
B2	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
B3	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
B4	0.0043	0.0043	0.0087	0.0043	0.0043	0.0043	0.0087	0.0043
AP10	0.0219	0.0219	0.0175	0.0219	0.0219	0.0219	0.0263	0.0219
K8	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
F7	0.0043	0.0043	0.0000	0.0043	0.0043	0.0043	0.0087	0.0043
H1	0.0043	0.0043	0.0087	0.0043	0.0043	0.0043	0.0087	0.0043
K21	0.0000	0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
I1	0.0000	0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
I4	0.0000	0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
I5		0.0000	0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
M21	0,0		0.0043	0.0000	0.0000	0.0000	0.0043	0.0000
F13	1,0	1,0		0.0043	0.0043	0.0043	0.0087	0.0043
M7	0,0	0,0	0,0		0.0000	0.0000	0.0043	0.0000
G1	0,0	0,0	0,0	0.0		0.0000	0.0043	0.0000
G2	0,0	0,0	1,0	1,0	1,0		0.0043	0.0000
G3	1,0	1,0	0,0	0,0	0,0	1,0		0.0043
G4	0,0	0,0	0.0	1,0	0,0	0,0	1,0	

Figure 6.--Cladogram constructed from Kimura-2 Parameter genetic distances for nine-banded armadillos, *Dasypus novemcinctus*. EI = island samples, AP = Arkansas Post, AR = Arkansas free ranging samples, and TX = Texas free ranging samples.


TX-G3
AR-M7
TX-G4
TX-G2
TX-G1
TX-I5
TX-I4
TX-I1
TX-K21
EI-F6
EI-F3
AR-M21
TX-H1
EI-BG2
AR-B4
Group 3
TX-K8
AR-B3
AR-F13
EI-F7
EI-BG3
AP-AP7
AR-B2
Group 2
AP-AP6
Group 1
AP-AP10
0.0%
0.01%
Genetic Distance Scale

DISCUSSION

The four populations examined here display low levels of genetic variability overall when compared to levels reported for other populations of mammals (Table 7). These results are in agreement with Huchon, *et al.* (1999) who reported low genetic variability in nine individuals of *D. novemcinctus* from Texas, Mississippi, and Louisiana. This previous study, based upon a sequence 492 nucleotides closer to the 5'-end of the D-loop, suggested populations of nine-banded armadillos exhibited a very low level of sequence diversity as a result of the founder effect. Moncrief (1988) reported an absence of genetic variation in, and Ramsey and Grigsby (1985) reported estimated overall heterozygosity values of 1.0% for, armadillos across the range of the species based on allozyme analysis. Typical heterozygosity values for all animals average about 9.0% in populations of vertebrates and invertebrates (Sealander and Kaufman, 1973; Lewontin, 1974).

Recently, a study conducted in the same area as the isolated population of the present investigation reported on mtDNA D-loop sequence variation in the Southern flying squirrel (*Gluacomys volans*). Sequence diversity was highest in the open population and was also relatively high in the island population (Cook, 1999).

Population Genetics

In the present study, when comparisons were made between populations, surprisingly, the Electric Island population did not appear to be genetically distinct when compared to both the Arkansas and Texas free ranging populations, thus the island population did not exhibit a founder effect. However, the semi-isolated Arkansas Post

population did appear to be genetically distinct from the other three. Even with the low sequence diversity in the four populations studied, three groups were indicated in all trees generated. These trees should be viewed with caution, as a total of only four nucleotide positions were phylogenetically informative.

With all clustering methods used, two of the three Arkansas Post specimens (AP6, AP10) were most dissimilar from all other specimens and are clustered in group 1 (Fig. 6). The third Arkansas Post specimen (AP7) clustered with two Electric Island specimens in group 2.

Table 7.-- Mitochondrial DNA D-loop sequence diversity for selected mammals.

ORGANISM	SEQUENCE DIVERSITY
Nine-banded armadillos- present study All	0.045%
Nine-banded armadillos-French Guiana United States (TX, LA, MS) All	1.0% 0.046% 3.05%
Flying squirrels-Electric Island and surrounding area All	 1.4- 4.00%
Pygmy opossums–isolated populations between populations	0.08-0.38% 0.7-1.53%
Bats (RFLP)-isolated populations between populations All	1.0-2.4% 0.8-1.9% 2.1%
Domestic dogs within taxa Wolves	2.06% 2.10%

(BG2, F7), the Electric Island/Mainland specimen (F13), and one Arkansas free ranging specimen (B2) in group 2. Individuals AP6 and AP10 had distinct haplotypes and represent the eastern and northernmost population studied. The Arkansas Post population is thought to be semi-isolated as it resides on a peninsula surrounded on three sides by water (river or swamp) with a bridge as the only means of access. Nine-banded armadillos colonized this area of Arkansas approximately 50 years ago. The animals could have stemmed from the Texas populations in the west, the Louisiana populations in the south, or theoretically across the Mississippi River (by bridge) from populations in the southeast populations. The Arkansas Post population shares some haplotype characteristics with the other populations, but the substitutions at 16166-G and 166167-C only appear in these two specimens. The unique haplotypes found at Arkansas Post represents a biogeographical anomaly worthy of further study.

The island population appears to be genetically similar to both free ranging populations. Two of the five specimens clustereded in group 2 and the three specimens grouped in group 3. An interesting note is that two Electric Island individuals (BG2, F7) did cluster in the same group as the only Electric Island/Mainland (F13) representative. However, as the haplotype shared by theses individuals only differed from the most common haplotypes by a single transition (16159-T), the genetic variation is considered to be minimal. The island population has been isolated from the mainland for approximately 70 years. Prior to the impoundment of the lake, nine-banded armadillos were approaching central Arkansas from the southwest (Selander and Heidt, 1990). In those 70 years, no discernable genetic differentiation has occurred and no founder effect

is evident for the Electric Island population. The clustering of these individuals into group 2 and the characters shared between both the island and free ranging populations brings up the question of dispersal. Dispersal between the island and the mainland peninsula was considered highly unlikely as the shortest distance between the mainland peninsula and Electric Island is 150 m. This distance is too far for nine-banded armadillos to swim. It would be possible, but highly implausible, that humans assisted in colonization of the islands.

Two of the Electric Island individuals shared the same haplotype as the mainland peninsula individual (F13), an Arkansas Post specimen, and a single Arkansas free ranging specimen. This specimen (F13) appears to have as much genetic variability as the free ranging population. The peninsula population is probably small and only one specimen was collected.

The Arkansas free ranging specimens have representatives in groups 2 and 3, whereas all of the Texas free ranging samples are located in a group 3. The amount of variation indicated for both of these populations supports the numbers reported by Huchon *et al.* (1999) for similar free ranging individuals. Some of these animals were separated by as much as 800 km, yet similar genetic values are reported. Huchon *et al.,* (1999) reported genetic diversity values for nine-banded armadillos specimens collected 32 km apart in French Guiana to fall within the range expected for placental mammals.

The continued expansion of nine-banded armadillos' range is well documented (Taulman and Robbins, 1996). Armadillos first appeared in South Texas in 1854 and have increased their range by four to 10 km per year since that time (Taulman and

Robbins, 1996). Hewitt (1989) suggested populations of rapidly colonizing organisms might experience decreased genetic variation at the front of the colonization waves. Colonization may produce a certain amount of genetic variation through a founder effect-type mechanism if the groups that found the new population are sufficiently small and homogenous (Hastings and Harrison, 1994). However, over time, drift should be accelerated and loss of heterozygosity should occur, both within and among populations. This may partially explain the lack of genetic variation reported in North American nine-banded armadillos. In addition to the effect of rapid colonization, the presence of monozygotic polyembryony may serve as a method of preserving the genetic make-up of such populations.

Reproductive Strategies and Genetic Variation

Polyembryony is a common reproductive strategy in parasitic wasps, some flatworms, and numerous aquatic invertebrates (Loughry, *et al.*, 1998). Within vertebrates, only the six mammalian species of *Dasypus* are polyembryonic. Craig, *et al.* (1997) suggested that the evolution of polyembryony might be explained as a means of increasing the number of offspring when sperm is limited or the female is under constraints for egg or embryo production. These would be adaptations to increase reproductive success due to certain reproduction constraints. If the latter constraint were selected, then Galbreath's (1985) hypothesis would agree:

> . . . some common ancestor of all extant armadillo species exhibiting polyembryony had a normal litter size of one. A specialized uterus evolved with a large fundic cavity, endometrial grooves, and a fundic apical depression. The adaptive value of permanently positioning a blastocyst in any area conducive to successful implantation might have been high if there was often a period of delay before implantation. This set of adaptations ensured that the blastocyst would be

positioned against the anterior section of the uterus.

Thus, implantation probability would be optimized. If the animal were polyzygotic, then the above scenario would disallow multiple successful implantation sites. Later, perhaps, a situation arose where a larger litter size was feasible and constraints of the uterus were bypassed by monozygotic polyembryony. Reproductive success increased and the strategy was favored by natural selection.

Also of interest is the presence of delayed and super-delayed implantation in the nine-banded armadillo. Super-delayed implantation (Storrs, 1988) resulted in delays of 12 to 24 months for five female nine-banded armadillos isolated from males for 16 months. Storrs suggested that this delay was in response to stress and is an unusual survival strategy that may aid in the explanation of the nine-banded armadillos' dramatic territory expansion. Extended delays in implantation may allow colonizing females to travel farther than they would have had implantation occurred within the typical time period. Again, it can be assumed that reproductive success increased and the strategy was favored by natural selection.

CONCLUSION

Waddell, *et al.* (1999) and others (*e.g.* Arnason *et al.*, 1997) have reported Xenarthrans (armadillos) are perhaps the slowest evolving of all placental mammals and have have based Xenarthran molecular evolutionary theory on North American nine-banded armadillo data. Perhaps their results would be different if the specimens analyzed came from the southernmost portion of this organisms' range as Huchon, *et al.* (1999) reported significant genetic variation between North and South American nine-banded armadillos.

The data presented here and elsewhere suggest that recent dramatic colonization of North America by nine-banded armadillos along with the presence of the reproductive strategies of monozygotic polyembryony and delayed implantation appear to have worked in concert to lower the levels of genetic variation across the North American range. Further investigation is warranted regarding populations in other regions of South America, Central America, and range limits in North America. If the above suggestion is true, then Central America should exhibit intermediary genetic variation, and the North American range limits should have even lower values than those reported in this study.

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